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SUMMARY DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY AND DISCUSSION

Renal aging is characterized by structural changes and functional decline. These changes make the elderly more vulnerable to chronic kidney disease, hypertension, and cardiovascular disease. Furthermore, they also make it more difficult to cope with stress factors, such as dehydration, toxicity, and obstruction. These stress factors can lead to acute kidney injury and reduced recovery from acute kidney injury and may result in chronic kidney disease or even end-stage renal disease. The rate of renal aging is influenced by environmental factors and lifestyle. In addition, several studies have provided evidence for the involvement of genetic background in the development of renal disease [1]). Genetic variation plays an important role in the differentiation between people at risk for accelerated renal aging and might explain why one-third of the aging population shows no functional decline [2].

In this thesis, several histological changes observed during renal aging have been analyzed in aged mice. Subsequently the associated genomic regions or candidate genes were identified and functionally investigated. The data presented may lead to a better understanding of the processes and pathways involved in renal aging and lead to identification of individuals at risk or even allow us to design therapeutic strategies that can delay renal aging.

An overview of the functional and structural changes observed during renal aging is given in chapter 2, followed by a review of the candidate genes involved in renal aging as identified by human GWAS and mouse genetic analyses. This chapter emphasizes that mice are important for mechanistic evaluation of candidate genes identified by human GWAS, but also for genetic screening, since mice have a high genetic resemblance to humans.

Histological changes of renal aging include interstitial fibrosis and tubular atrophy, vascular intima fibrosis, and sclerosis of the glomerulus. Mesangial matrix expansion (MME), one of the precursors of glomerulosclerosis, is caused by an imbalance between the formation and breakdown of the extracellular matrix. Chapter 3 deals with the identification of genes involved in the susceptibility for MME. For this purpose, MME was characterized in 24 inbred mouse strains at 6, 12 and 20 months of age. The gene *Far2* was found to be associated with the presence of MME and sequencing revealed a 9-bp insertion in the 5'untranslated region of *Far2* associated with the presence of MME at 20 months. Furthermore, both *in vivo* and *in vitro* this insertion was associated with an increased expression of *Far2*. However, there were some strains in our study with the *Far2* insertion in which MME was not observed. Most likely, additional loci in the mouse affect MME where the unaffected strains have alleles that counteract the *Far2* allele. However, our current study lacks the power to identify these loci. Overexpression of *Far2*

in vitro produced an upregulation of MME-promoting factors; platelet-activating factor (PAF) and TGFβ. *Far2* encodes the protein fatty acyl-coenzyme A reductase 2, that catalyzes production of fatty alcohols, i.e. precursors of PAF. Based on these data we propose that *Far2* is involved in renal aging via the PAF/TGFβ - pathway. The role of PAF in the kidney and its production by, and actions on, mesangial cells have recently been reviewed [3]. PAF is involved in pro-inflammatory and pro-fibrotic processes, which fits well in our proposed pathway. Furthermore, it was demonstrated that the receptor for PAF (PAFR) shows the highest expression in the glomerulus and knockout of PAFR in an unilateral ureter obstruction model showed reduced profibrotic signaling and collagen deposition [4,5].

A strategy to reduce age-related MME is to target PAF by antagonizing the PAFR or increasing the enzymatic breakdown of active PAF. Another strategy is to directly intervene in the expression or activation of *Far2*. However, little is known about specific functions of *Far2* in mammals. Since inhibition of *Far2* might have unknown consequences, we should first evaluate the effects of a systemic knockdown of *Far2* on the kidney and other organs.

Chronic inflammation is known to influence renal aging by several mechanisms, including the production of pro-fibrotic factors and oxidative stress with the generation of advanced glycation end products, which in turn further stimulate inflammation and renal aging. In chapter 4, perivascular immune-cell clusters were characterized in the same cohort of aged mice as in the previous chapter. The clusters consisted of leukocytes, primarily T cells and to a lesser extent of B cells, and were shown to increase in relative cluster size with age. Eventually these clusters developed into tertiary lymphoid organs (TLO), as shown by the presence of proliferating cells, high endothelial venules, and lymph vessels. This process was not specific to the kidney since a similar process could be observed in the liver. Haplotype association identified several genomic regions that were significantly associated, but different for male and female mice. In male mice, the strongest association was found with a locus on Chr 2, containing the gene *Wisp2*. In female mice, the haplotype blocks containing *Ctnnbip1* and *Tnfrsf8* showed the strongest association. *Wisp2* and *Ctnnbip1* are part of the Wnt signaling pathway, while *Tnfrsf8* is expressed in activated T and B cells. Coding differences in exon 4 of *Wisp2* and exon 5 of *Tnfrsf8* were identified, although the latter does not show a clear difference between strains with and without perivascular immune cell clusters. A Q-allele in exon 4 of *Wisp2* could promote the development of immune-cell clusters, since all strains with a cluster size above the threshold had this allele. However, four strains had the Q-allele but had no perivascular cell clusters, and in this case other genomic loci could be involved. Another locus significantly associated with the phenotype in male mice was identified on Chr 1, containing the gene *Esrrg*.

Although the presence of TLOs was not correlated with a decline of renal function, we cannot exclude an increased vulnerability to renal damage in response to a second hit. Therefore, it would be interesting to further study intervention in the Wnt signaling pathway via *Wisp2* and *Ctnnbip1* or to use immunosuppressives to downregulate the immune response and observe the size and presence of TLOs with increasing age.

Upon screening of the kidneys of the 20-month-old mice, we observed an interesting phenotype in some strains; the presence of glomerular intracapillary deposits. In chapter 5, we reported that these deposits are present in six mouse strains at 20 months of age, while they were absent or less extensive in the younger mice at 12 and 6 months of age. The glomerular deposits were further characterized by electron microscopy and immunohistochemistry, which demonstrated that the deposits contain lipoproteins. Genetic analysis identified a 30-Kb region, within the *Esrrg* gene, on Chr 1 associated with these glomerular deposits. Interestingly, *Esrrg* is known to influence lipid transport and metabolism and thus could be involved in the evolvement of these glomerular deposits. However, we could not detect any differences in Essrg mRNA or protein expression between strains with or without glomerular deposits. Furthermore, we observed a high variation in severity of deposits between individual mice within strains. Glomerular deposits were considered a very complex phenotype, in which other loci and environmental factors are very likely involved. Since only a few strains showed the presence of glomerular deposits it is difficult to find a correlation with other genomic loci involved.

This glomerular phenotype resembles the human disease lipoprotein glomerulopathy (LPG), which is characterized by proteinuria, abnormalities in serum apolipoproteins, and occlusion of glomerular capillaries by lipoprotein-containing material. LPG can progress to end-stage renal disease [6-8]. Lesions are localized to glomeruli, which could be due to the micro-environment of the renal glomerulus which might favor formation of lipoprotein aggregates within the capillaries. However, LPG is not known as an age-related disease, since it can affect all age groups and is not dependent on intrinsic renal abnormalities since recurrence is observed after renal transplantation. The pathogenesis of LPG is unclear, although alteration in ApoE function and structure might play a role. Various ApoE gene mutations can predispose for the development of lipoprotein glomerulopathy, but also in humans there are asymptomatic carriers, suggesting that other genetic and epigenetic factors are involved [9]. Unfortunately, cholesterol and albumin-to-creatinine (ACR) ratios that corresponded exactly to the mouse-kidney samples analyzed were not available, so potential correlations between these parameters and the phenotype could not be observed. However, based on the strain average data reported in the phenome database, no consistent

increase in ACR or non-HDL levels for the affected strains compared to the strains without deposits is seen.

Treatment of LPG patients with fenofibrate (a lipid-lowering drug) reduces proteinuria and serum apolipoprotein abnormalities, but most importantly causes remission of glomerular lesions, even in patients with normal lipid values [9]. Therefore, it would be interesting to treat affected animals with a lipid-lowering drug to evaluate its renoprotective effects, and evaluate whether it can prevent the evolvement of glomerular lipoprotein deposits. Interestingly, of a study into the effect of fenofibrate on genes of lipid and lipoprotein metabolism in human ApoA-I transgenic mice, a significant upregulation of *Esrrg* was observed [10]. In summary, the literature indicates that glomerular lipoprotein deposits are associated with lipid levels and ApoE polymorphisms, lipid-lowering drugs (fenofibrate) can reverse this phenotype, and the effect of these drugs might be regulated by the transcription factor Estrogen-related receptor gamma. Future studies on the involvement of *Esrrg* in evolvement of glomerular deposits should include a knockdown of *Esrrg* specific for the kidney in affected strains and observe if it is still possible to evolve glomerular deposits and investigate which other loci might be involved.

Interestingly, Estrogen-related receptor gamma was identified as an associated gene with perivascular infiltrates (chapter 4) and glomerular deposits (chapter 5). The identification of *Esrrg* associated with these two phenotypes suggests that *Esrrg* variance is associated with the development of both phenotypes in male mice, and that these phenotypes might have a similar underlying pathophysiology. There is a previous study that reported on an increase of perivascular infiltrates with age, as well as the presence of glomerular deposits recognizable from the age of 3 months onwards, growing larger with age [7]. This phenomenon was observed in NON mice, the only strain which we observed positive for both glomerular deposits and immune cell clusters. Furthermore, a similar interval on Chr1, containing *Esrrg*, was found associated with cGVHD susceptibility and increased CD4+ T cell intrinsic activation in an NZW-derived strain [11]. Therefore, the underlying mechanism might be related to alterations in the immune response associated with aging, leading to glomerular deposits and immune-cell clusters.

Although we can only speculate on the role of *Esrrg* in the evolvement of glomerular lipoprotein deposits and perivascular infiltrates, we concluded that *Esrrg* is a gene significant for renal aging phenotypes. This is further supported by a study that identified genes that were differentially expressed in the kidney with age, among which *Esrrg* showed a 1.4-fold decrease with age [12].

Esrrg is an orphan nuclear hormone receptor expressed in tissues with high metabolic activity like the heart and kidney. *Esrrg* is involved in the regulation of

hepatic gluconeogenesis and there is a correlation between certain SNPs and altered blood-pressure [13,14]. SNPs associated with hypertension and lower systolic blood-pressure were identified, suggesting a critical role for *Esrrg* as both a positive and negative influence on blood-pressure [13,14]. In chapter 6, the biological function of *Esrrg* in the kidney was further investigated *in vitro* and *in vivo*. Based on the co-localization of ERRγ and renin in the afferent arteriole in the glomerulus we hypothesized that *Esrrg* is a regulator of renin expression. We confirmed this hypothesis by stimulating renin-producing cells with an *Esrrg* agonist, which led to increased renin production. Stimulation of the renin promoter with this *Esrrg* agonist induced a three-fold increase in renin-promoter activity. Although knockdown of *Esrrg* with siRNAs decreased renin-gene transcription and renin-promoter activity after agonist stimulation, it did not completely abolish the effect of the agonist. This could be due to incomplete knockdown of *Esrrg* or the involvement of other (nuclear hormone) transcription factors or co-activators. Although we did not show direct binding of the gene to the renin promoter, this is something we will definitely focus on in the near future.

Esrrg might be involved in kidney aging through the regulation of renin production. With advancing age, a decline in renin plasma activity is observed. This physiological decline may act to reduce the availability of Angiotensin II, which has several detrimental age-accelerating effects in the kidney [15,16]. The decrease of renin with advancing age might be regulated through *Esrrg*, which was also demonstrated to decline with age.

FUTURE PERSPECTIVES

The geriatric population is increasing, therefore it is important to unravel the mechanism of renal aging and identify underlying pathways. Several studies show evidence for the genetic basis of renal disease, meaning that a certain genetic variation can lead to accelerated renal aging. Human GWAS have identified several genes associated with renal disease, of which some might have an important role in renal aging as well. Although genetic variations have been identified, it has been proven difficult to identify the causative variants or explain the underlying biological mechanism.

We should combine the power of human and animal studies. Since possibilities for testing in humans are limited, mouse and other animal models should be used to explain the underlying biological mechanism of genes and loci that are identified by human GWAS. We should leverage the power of mouse models, not only to explain the pathological mechanism, but also to identify candidate genes involved in renal aging. Mice share 99% of their genes with humans. In addition, mouse studies are more time- and cost-effective than human studies,

environmental factors can be controlled, and it is possible to analyze histological changes in more detail. Focus on histological changes, not far downstream of the disease cascade, which might even make detection of subtle changes possible before they actually affect kidney function. Despite the many advantages of using mouse models we should be aware of the differences between mice and humans. Manipulations that generate renal damage are difficult to induce in mice, they show a great sensitivity to stress and infection and due to their small size surgical procedures are more difficult than for example in rats [17,18].

This thesis provides several examples of mouse-strain usage to identify candidate genes associated with renal aging by using haplotype association mapping to further explore the biological function of specific candidate genes.

In the near future even higher resolution genetic mapping is being developed by creating a diversity outbred population with higher genetic diversity [19]. While controlling environmental and population factors, we will have access to renal tissues at specific time points for histology, proteomics, and gene expression per individual mouse. Susceptibility genes in outbred strains might produce more variable phenotypes, closer to human disease.

In the future, we should not only focus on aging of the kidney, but examine aging effects in different organs as well and investigate if the same aging processes are involved. In this way we can determine if a certain process is specific to the kidney or common to the general aging process. For this purpose, collaboration between disciplines in lung, heart and kidney research is very important.

In this thesis, we have identified candidate genes associated with age-related mesangial matrix expansion, perivascular immune-cell clusters and glomerular lipoprotein deposits. In the future, other aging-related phenotypes, such as glomerulosclerosis, interstitial fibrosis, and vascular intima fibrosis should be characterized and genetically analyzed. The phenotypes in this thesis and other aging-related phenotypes should be characterized in a recently developed mouse population with higher genetic diversity. Higher resolution mapping can help us to identify genetic loci more precise or identify counteracting alleles. Furthermore, functional follow-up of the candidate genes identified in this thesis, especially *Far2* and *Esrrg*, should be performed to further unravel the underlying pathway and design therapeutic interventions.

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